STIC-ILL 4267819 Vol no 362402

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The Prostate 21:87–97 (1992)

Presence of Calcitonin-Like Immunoreactivity (iCT) in Human Prostate Gland: Evidence for iCT Secretion by Cultured Prostate Cells

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Immunoreactive calcitonin (iCT) has been detected in human prostate tissue extracts as well as seminal plasma. The present studies were undertaken to examine whether iSCT (immunoreactive salmon CT-like human peptide) co-exists with iHCT (thyroid CT-like substance) in human prostate tissue extracts, and whether these substances are secreted by primary prostate cells in culture. Since the local secretion of these substances seems to increase in some neoplasms, a second objective of the study was to examine whether basal secretion of iCTs from primary prostate cells is increased in carcinoma. The present results have shown that both iHCT and iSCT were present in prostate tissue extracts. The mean iHCT levels in extracts of benign hyperplastic prostates (BPH) were 0.59 ng/g prostate, and these were significantly lower than iHCT concentrations in prostatic carcinoma (PC) (2.53 ng/g). No significant differences in their iSCT contents were observed. However, the results from culture of over 90 individual prostate tissue specimens from BPH or PC indicate that primary prostate cells secreted detectable quantities of iSCT and the basal release of this material from PC prostate cultures was almost four-fold higher than that from BPH prostate cultures.

These results suggest that a CT-like immunoreactive material is secreted by primary prostate cells in culture, and the basal secretion of this material is significantly higher in PC cells as compared to BPH cells. Endogenous secretion of prostatic CT, and the elevation of its expression in PC suggest that it may serve as a regulatory factor in the pathophysiology of the prostate gland.

Key words: calcitonin, prostate cells, culture, BPH, prostatic carcinoma

INTRODUCTION

Calcitonin (CT), a 32-amino acid peptide hormone, is secreted by parafollicular or C cells of the thyroid gland. It inhibits bone resorption and increases renal clearance of sodium, phosphate and calcium [1]. In addition to CT, various related pep-

Received for publication December 11, 1991; accepted April 6, 1992.

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tides or gene products have been detected in several organs and body fluids. For example, CT gene-related peptide (CGRP) is produced in neuronal cells by alternative processing of the primary CT gene transcript. Two different CGRP peptides, CGRP-α and CGRP-β, have been described in the human and rat brain, and seem to be products of two different CT genes [2]. Ketacalcin, a carboxy-terminal flanking peptide of the CT-precursor, has also been identified in body fluids, and is considered as a secretory product of CT gene [2]. In addition, the presence of a peptide referred to as salmon (S)CT-like peptide has been demonstrated in humans and several other mammalian species, and its presence in normal human sera, milk, the brain, and the thyroid gland has been demonstrated by specific antibodies [3–6]. Besides their predominant presence in the thyroid gland, CT and related peptides are also present in large concentrations in seminal plasma, and high affinity binding sites for SCT are identified in the neck and mid-piece region of human spermatozoa [7–10]. This suggests that CT and related peptide(s) may have a physiological role in regulation of sperm activity and other male reproductive processes.

Seminal CT levels in split ejaculate correlate with zinc and other prostatic secretions [10]. These findings indicate that CT in seminal plasma may originate from the prostate gland. Subsequent studies have shown that measurable quantities of immunoreactive CT are present in prostate tissue extracts, and the CT-immunopositive cells have been localized among neuroendocrine cell populations of the prostate gland [11–15]. Accumulated CT levels in tissue extracts of benign hyperplastic prostate (BPH) are significantly lower than normal prostate tissue [12]. In contrast, relative population of CT-immunopositive and other neuroendocrine cells increases with progression of prostatic adenocarcinoma (PC) [15]. This raises a possibility that alterations in prostate CT expression may have a functional significance in the pathophysiology of the prostate gland. However, there is no direct evidence for biosynthesis of CT or related peptides in the prostate gland, or for whether their local secretion is altered in prostate dysfunction.

Using recently developed specific and sensitive RIAs for HCT and SCT [6,16], we have investigated 1) whether a SCT-like peptide co-exists with HCT in seminal plasma and prostate tissue extracts, 2) whether CTs are synthesized and released by benign and malignant prostate cells, and 3) whether the rate of their basal secretion is altered in malignancy.

MATERIALS AND METHODS

Prostate Tissue Specimen

Human prostatic tissue specimens obtained were either from biopsy or from freshly resected glands removed during transurethral prostectomy or radical prostatectomy. The surgeries were performed at the University of Kansas Medical Center and the Kansas City Veterans Administration Hospital. The age of this patient population ranged from 48 to 71 years (mean age: 63 years). The patients did not receive any hormonal or drug treatment prior to the collection of tissue specimens. Appropriate informed consent was obtained in all cases.

Semen Samples

Semen samples were obtained from healthy male volunteers with normal spermiograms. The samples were obtained in polyethylene cups after 3–5 days of absti-

nence. The samples were allowed to liquify at room temperature for 30 min, were centrifuged to remove sperm and sediments, and were stored frozen at −20°C.

Extraction of Seminal Plasma and Prostate Tissues for RIA

Semen and prostate tissue specimens from BPH and cancer patients were processed using a modification of the method of Tobler et al. [17]. In brief, after determining the wet weight of frozen tissues (40–110 mg), tissues or seminal plasma samples were suspended in 2 M acetic acid (1:20 wt/vol for prostate tissues and vol/vol for seminal plasma) and heated at 85°C for 5 min. The tissues were homogenized in a dounce homogenizer, and resulting supernatants were centrifuged at 36,000 rpm for 30 min at 4°C. The clear supernatant was passed 20 times through Sep-Pak C_{18} cartridges (Waters associates, Milford, MA) activated with 10 ml methanol and equilibrated with 20 ml 0.1% trifluoroacetic acid (TFA) in water (vol/vol). The cartridges were washed with 40 ml 0.1% TFA, and CT fraction was eluted in 15 ml 80% methanol in water containing 0.1% TFA. The samples were lyophilized after addition of 10 ml 0.1 M acetic acid. Our previous studies suggest that the recovery of exogenously added SCT and HCT in a sample by this method ranges between 75 and 106% [6].

Tissue Culture

Needle biopsy tissue and transurethrally resected prostate chips were collected aseptically and placed individually in sterile transport medium [RPMI-1640 without fetal bovine serum (FBS)]. These fragments were transported to the laboratory, a portion of these fragments were fixed and frozen for immunohistochemistry, and the remaining fragments were individually cultured as described before [18,19]. In brief, the tissue was cut to remove the burned areas and minced into 1 × 2 × 1 mm sections. Approximately, eight of such sections were evenly distributed in a 25 cm^2 tissue culture flask (Falcon, Oxnard, CA). Culture medium, 1.0 ml (basic RPMI-1640 medium supplemented with L-glutamine, 10% newborn calf serum, 25 mM Hepes, 10 ng/ml epithelial growth factor, 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml selenium, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml 5 α-androstan-17-ol-3-one), was then gently added to each flask so as not to shake the explants off, and the explants were allowed to plate for 7 days at 37°C with 5% CO_2 in a humidified atmosphere. After the initial 1st week, the culture medium was replaced twice every week until the flask became confluent with cells. Under our experimental conditions, it takes approximately 5 to 6 weeks for the cells to grow to confluency. Separate pools of media were prepared for feeding each patient's cells to avoid risk of cross-contamination. At confluence, the cells were washed and the culture medium was replaced with basal incubation medium (basic RPMI 1640 containing 1-glutamine, 0.3% BSA, 10 mM Hepes, gentamicin, and bacitracin). The cells were incubated for 24 h and the spent media were collected and stored at −80°C until assayed for HCT and SCT contents by RIA. The cells (viability > 90% by trypan blue exclusion) were then harvested and counted with a hemocytometer. Our earlier studies suggest that a confluent 25 cm^2 tissue culture flask contains approximately 0.5–0.6 million primary prostate cells.

Radioimmunoassays

Both HCT and SCT RIAs employed a similar non-equilibrium protocol as described before [6,16].

Synthetic human CT (Peninsula Laboratories, Belmont, CA) was iodinated with ^{125}I to a specific activity of 33 μCi/μg with chloramin T, and was purified as described before [17]. Anti-HCT rabbit serum (LD-2, kindly provided by Dr. L. Deftos, University of California, San Diego) was used at a dilution of 1:3,500. The assay provides a detection limit (2 SD from B_o) of 2 pg/tube using synthetic HCT as a reference preparation. SCT RIA was essentially the same as described before [6]. The detection limit of the assay is 8–16 pg/tube of synthetic SCT. Anti-SCT rabbits serum (GCT1) was obtained following immunization with synthetic SCT (1-34)-keyhole limpet hemocyanin conjugate [6]. No cross-reaction of HCT and SCT occurred in two homologous RIAs in amounts as high as 75 ng of either peptide, and neither anti-serum recognized CT gene-related peptide. Further characterization of the antisera has been described previously [6,16].

All seminal plasma or prostate tissue extracts as well as spent media from cultured prostate cells were analyzed for the presence of HCT-like and SCT-like immunoreactivity. The results are expressed as ng CT Eq/g tissue or ml seminal plasma and pg CT Eq released per million cells. Intraassay variations for the assays were determined by running selected specimens in at least six to eight replicates. Parallelism between reference preparations and a pool of seminal plasma extracts or culture media was examined by running serial dilutions of the samples with serial dilutions of synthetic HCT or SCT in an assay.

Immunocytochemistry

Dispersed prostate cells were stained for SCT immunocytochemistry according to the modified method of Denef et al. [20]. Cells (5×10^5) suspended in 100 μl RPMI were allowed to attach for 24 h (at 37°C) to a poly-L-lysine coated glass coverslip placed in an individual well of a 6-well culture plate. After the incubation, the cells were fixed for 30 min (at room temperature) with 4% formalin in PBS (pH 7.3), washed three times in PBS, and stored in PBS (at 4°C) containing 5% normal goat serum. For immunostaining, the cells were incubated with 3% H_2O_2 for 10 min at room temperature to inactivate the endogenous peroxidase activity. The cells were then incubated sequentially with 1) blocking solution (PBS containing 1% bovine serum albumin, 0.3% Triton X-100, 0.2% nonfat dry milk, 0.01% sodium azide) for 10 min at room temperature; 2) anti-SCT serum (1:500, diluted in the blocking buffer) overnight at room temperature; 3) goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel Laboratories, West Chester, PA; diluted 1:20 in the blocking buffer) for 1 h at room temperature; 4) peroxidase-antiperoxidase incubation (Cappel Laboratories; 1:80 in the blocking buffer) for 1 h at room temperature. The color was developed with 10 mg diaminobenzidine tetrachloride in Tris-NaCl containing 0.06% H_2O_2 for 30–40 min.

A parallel set of control slides was processed similarly, but in place of primary anti-SCT serum, the slides received equivalent amounts of either preimmune serum or anti-SCT serum preabsorbed with 10 μM SCT (peptide control). Staining with anti-HCT was not performed, since the results from the culture experiments indicated a lack of detectable iHCT release by prostate cells.

Statistical Analysis

The data were analyzed by analysis of variance and the statistical significance was derived from Student's t-test.

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TABLE I. Concentrations of Immunoreactive Calcitonins (iHCT and iSCT) in Seminal Plasma and Prostate Tissue Extracts

	iHCT ($\bar{X}$ ± SEM)	iSCT ($\bar{X}$ ± SEM)	
Seminal plasma (ng/ml)	0.78 ± 0.28	0.048 ± 0.023	n = 4
Prostate extracts (ng Eq/g tissue)			
BPH	0.59 ± 0.16*	0.98 ± 0.19	n = 10
(range)	(0.16–1.65)	(0.39–1.89)	
PC	2.53 ± 0.97*	0.98 ± 0.18	n = 9
(range)	(0.24–10.63)	(0.20–1.73)	

*Significantly different, $P < .05$ (paired t-test).

RESULTS

Presence of CTs in Prostatic Tissue Extracts and Seminal Plasma

The detection limit of HCT RIA (2 SD from B_o) was 1.95 pg of synthetic HCT and the intraassay variability was less than 5%. In the initial assay, a pool of seminal plasma extracts was serially diluted with synthetic HCT, and the extract exhibited parallelism with HCT suggesting a similarity in the antigenicity of seminal HCT and synthetic HCT (data not shown). Likewise, a dilution curve of this extract also exhibited parallelism with a dilution curve of synthetic SCT in a SCT RIA. Subsequently, all prostate tissue and seminal plasma extracts were analyzed in a single assay to avoid interassay variability. We could not obtain normal prostate tissue specimens for the assay, but altogether 19 diseased prostate tissues [prostate cancer (PC)-9; benign prostatic hypertrophy (BPH)-10] and 5 seminal plasma samples were extracted and analyzed for CTs by RIA. The results are presented in Table I.

All prostate tissue and seminal plasma extracts contained detectable quantities of HCT-like (iHCT) as well as SCT-like immunoreactivity (iSCT), and exhibited a wide range in their peptide content. While HCT content in BPH tissues was significantly lower than extracts from PC, there were no significant differences in their iSCT content (Table I). The levels of iHCT in tissue extracts and seminal plasma in the present study are comparable to previously reported results of others [8,12].

Release of CTs by Primary Prostate Cells

In all, 91 primary prostate cells from individual BPH or PC specimens were grown to confluence. The cells were incubated with the basal medium for 24 h, and the spent media were analyzed for CTs by RIAs.

The spent media samples from individual prostate cultures were analyzed for HCT, and none of the 91 cell culture media samples exhibited detectable amounts of iHCT. Similar results were obtained when these samples were reassayed with a commercially available HCT RIA kit (INC-star, Stillwater, MN).

In contrast to the absence of detectable amounts of iHCT, a majority of the culture media samples contained measurable quantities of iSCT, and a dilution curve of pooled culture media was parallel to the displacement curve of synthetic SCT (Fig. 1). Of the total 91 cell culture media tested, iSCT levels in 22 samples were below the detection limit of the assay. Eighteen of these 22 samples were from BPH prostates, while the remaining 4 were obtained from PC. The results presented in Figure 2 show that prostate cancer cells released a mean of 114 pg iSCT Eq/million

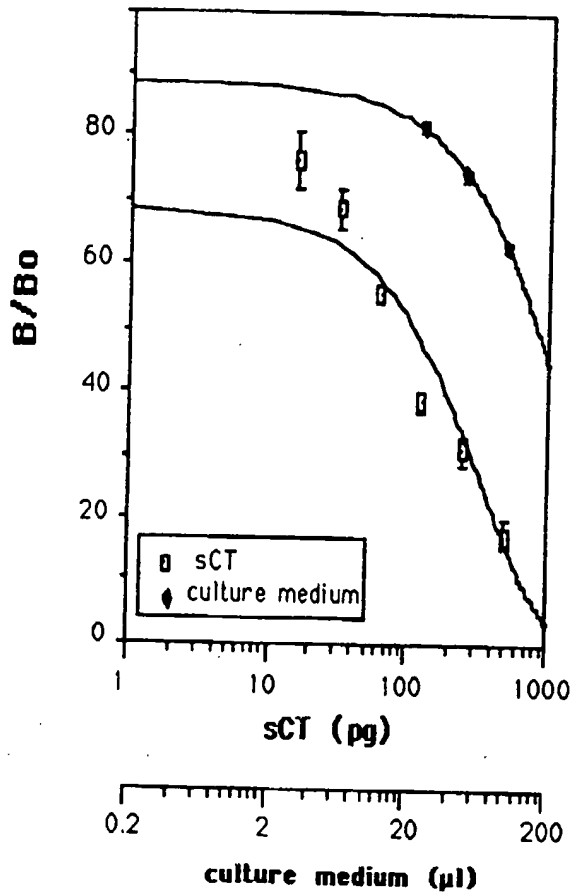


Fig. 1. Parallel displacement of ^{125}I-SCT binding to anti-SCT by synthetic SCT and prostate culture media in a SCT RIA.

cells/24 h, which is significantly higher than 34 pg iSCT Eq released by cells obtained from BPH prostates ($P < .05$, unpaired t-test).

Immunocytochemistry

At the end of the cell culture experiment, the prostate cells were dissociated from the culture flask. The cells exhibited a typical spherical appearance and good viability (>90%). Immunocytochemical study indicates that a small population of cells stained positively for iSCT, and as expected, staining in the immunopositive CT cells was localized to the cytosol (Fig. 3). Neither preimmune serum control nor peptide control exhibited significant staining (data not shown).

DISCUSSION

The presence of high concentrations of immunoreactive HCT in human seminal plasma and prostate tissue extracts has been demonstrated, and the present results are consistent with these findings [7–12]. In addition, the present study reveals the presence of SCT-like immunoreactivity in prostate and seminal plasma extracts. A

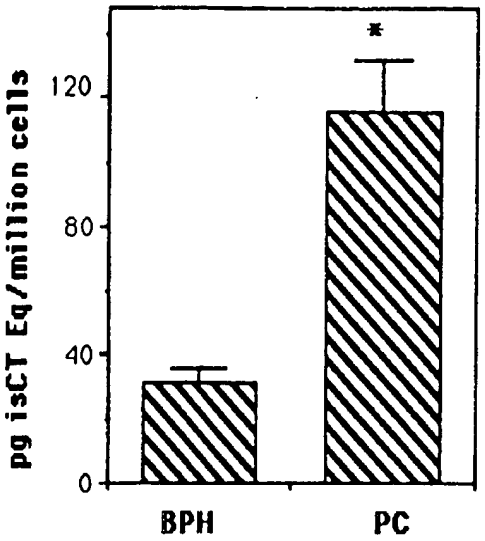


Fig. 2. Immunoreactive SCT concentrations in culture media from primary prostate cells obtained from prostatic carcinoma (PC) and benign prostatic hypertrophy (BPH). Each data point represents the mean ± SEM of n samples ($n_{BPH} = 41$; $n_{PC} = 50$). *$P < .05$ (comparison between BPH and PC by unpaired t-test).

similar peptide has been detected in normal humans as well as in patients with small cell carcinoma of the lung [4,5,21,22]. While this peptide is present only in trace amounts in the thyroid, it is more abundant in human brain [4]. Although the precise sequence of SCT-like human peptide remains to be determined, the peptide immunoreacts with the antiserum raised against SCT, co-elutes with synthetic SCT on reverse phase high performance liquid chromatography, and messenger RNA preparations from the thyroid gland of medullary thyroid carcinoma patients direct the synthesis of an anti-SCT serum precipitable peptide in vitro [4,22]. Binding sites recognizing salmon, but not human, CT have been recognized in various regions of brain as well as human spermatozoa, and ^{125}I-SCT also binds with high affinity to CT receptors in the bone and the kidney [23–25]. The presence of iHCT and iSCT in seminal plasma suggests that the peptides may have a role in regulation of sperm activity and other processes leading to fertilization. However, any direct effect of HCT on sperm activity or semen quality has not been suggested [7,8,24]. In contrast, SCT specifically inhibits sperm motility at subnanomolar-nanomolar concentrations. This action of SCT seems to be receptor-mediated and specifically affects calmodulin-dependent intracellular mechanisms [24]. Thus, the presence of iSCT in seminal plasma suggests that it may serve as an endogenous ligand for SCT-receptors on human spermatozoa and regulate fertility by preventing premature activation of human spermatozoa.

In addition to their potential role in regulation of spermatozoal activity, CT-like peptides may have a role in the physiology and pathophysiology of the prostate gland. Similar to the previous findings of others, the present results suggest that accumulated iHCT content in hyperplastic prostate is significantly lower than malignant prostate tissue [18]. However, iSCT levels were similar in both groups. Since the tissue specimens in the present study were collected from several patients of varied age and

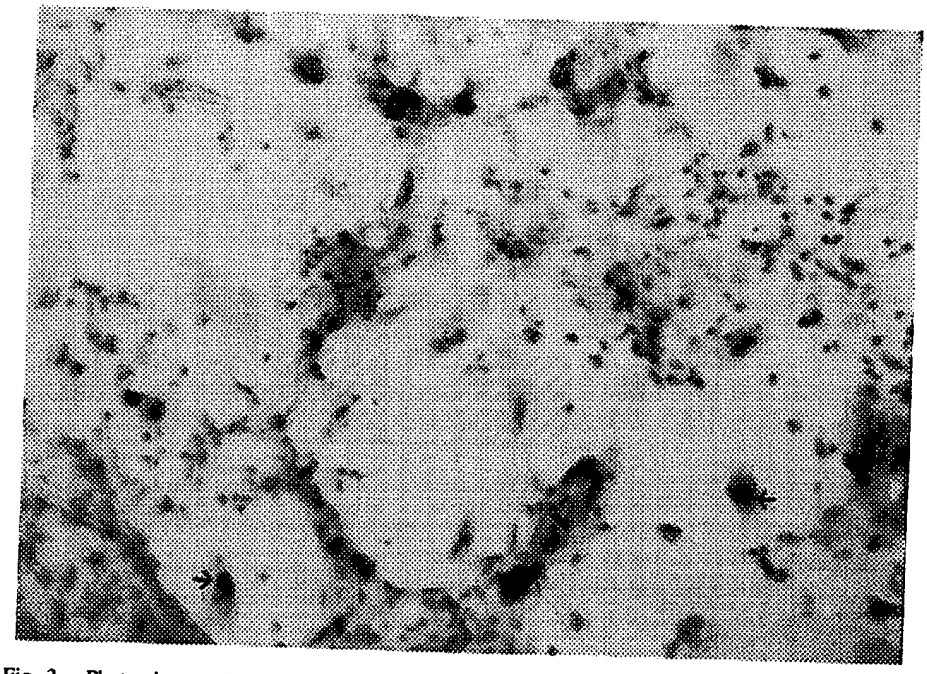
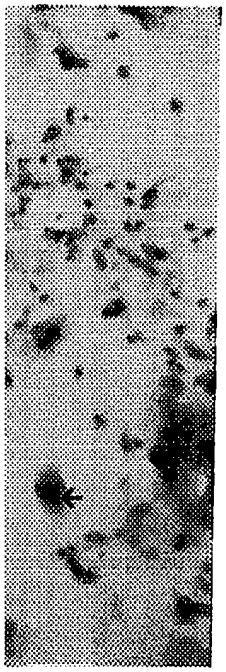


Fig. 3. Photomicrograph of cultured prostate cells immunostained with anti-SCT serum. Dark immunoperoxidase-stained CT-immunopositive cells (indicated by arrow) are distributed among lighter, hematoxylin-stained prostate cells. The cells had round or fusiform cell bodies, and some had extended neurite-like processes. Magnification: ×200.

health status, these results may have been influenced by wide individual variations in tissue and serum iCTs in patient populations [26]. To examine whether iCTs detected in tissue extracts are of prostatic origin or are acquired from circulation, we have tested their presence in secretions from primary prostate cultures. Since cells from individual prostate specimens were cultured under identical conditions, and tested for CT release after several weeks of culture and several media changes, concentrations of CTs released into the medium during the test period may indicate their endogenous secretory activity. The results indicate the absence of detectable iHCT in spent media, and suggest that iHCT in prostate tissue extracts may have been derived from circulation. Alternatively, iHCT-secreting prostate cells do not survive under our experimental conditions. In contrast, iSCT was present in the majority of the prostate culture media, and its secretion from cancer cells was almost four-fold higher than benign prostate cells. Thus, the initial findings suggest that the endogenous CT-like immunoreactive material secreted by primary prostate cells shares antigenic sites with SCT. Moreover, the extent of increase in iSCT secretion by cancer cells seems similar to that seen in prostate tumor markers such as prostate-specific antigen (PSA) and prostate acid phosphatase (PAP) [27]. Increased iSCT secretion has also been reported from lung cancer cells, and this was found to be parallel to other tumor markers such as carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE) [21]. This raises a possibility that prostatic CT may serve as a novel tumor marker for



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CT has been detected in numerous extrathyroidal tissues such as the brain, the gastroinestinal tract, the lungs, and the prostate gland [28]. Considering the presence of CT in diverse organs, it is conceivable that thyroid CT may be largely responsible for the maintenance of Ca^{2+} homeostasis and skeletal structure, whereas locally synthesized CTs may serve as paracrine regulatory peptides in respective organs. CT gene products are also expressed by several non-thyroid human neoplasms, and expression of these products as well as CT receptors seem to increase with progression of the tumors [28–31]. This suggests that CTs may play a role in regulation of growth or secretory function of neoplastic cells, and various growth-promoting effects of CT support this possiblity. For example, in vivo administration of SCT induces testicular, brain, and renal ornithine decarboxylase, a key enzyme associated with growth processes and cell cycle progression [32]. Recent evidence has shown that the action of CT in a target cell may vary according to the position of the cell in the cell cycle. For example, CT stimulates ouabain-sensitive Na^+-K^+ adenosine triophosphatase in pig kidney cells when the cells are in G2 phase, and inhibits this enzyme when the cells are in S phase [33]. These cell cycle-linked and biologically opposite actions of CT in the same cell suggest that the peptide may play an important regulatory role during periods of rapid growth such as entry of a quiescent cell into cell cycle and cell proliferation. Similar actions of CT in prostate cells may be of importance in regulation of growth during tumor formation and tumor growth.

In conclusion, the present study has shown that primary prostate cell secrete detectable amounts of CT-like immunoreactivity (prostatic CT) in culture. The cultures from prostatic carcinoma secrete significantly higher quantities of prostatic CT as compared to the cultures from BPH prostates. These findings suggest that prostatic CT may serve as a novel tumor marker for prostate cancer. Endogenous secretion of prostatic CT, coupled with the presence of its high affinity binding sites in the prostate gland (Shah et al., in preparation) and spermatozoa, raise a possibility that prostatic CT may play a paracrine regulatory role in the pathophysiology of the prostate gland and also affect sperm activity. Further studies are necessary to identify this product and to understand the regulation of its secretion and biological effects in the prostate gland.

ACKNOWLEDGMENTS

The authors thank Ms. Vicki Edelman and Mr. Mukund B. Jadhav for expert technical assistance, and Ms. Jeanne Selby for secretarial help. This work was supported by SEP grant from Marion Merrel Dow and the NCI, NIH, and the Department of Veterans Affairs (L.J.D.).

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